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Evidence for biased use of sperm sources in wild female giant cuttlefish (*Sepia apama*)

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In species where females store sperm from their mates prior to fertilization, sperm competition is particularly probable. Female *Sepia apama* are polyandrous and have access to sperm from packages (spermatangia) deposited by males onto their buccal area during mating and to sperm stored in internal sperm-storage organs (receptacles) located below the beak. Here, we describe the structure of the sperm stores in the female's buccal area, use microsatellite DNA analyses to determine the genetic diversity of stored sperm and combine these data with offspring genotypes to determine the storage location of paternal sperm. The number of male genotypes represented in the sperm receptacles was significantly lower than that found among the spermatangia. Estimation of the volumes of sperm contained in the receptacles and the spermatangia were statistically comparable; however, paternal sperm were more likely to have come from spermatangia than from the sperm receptacles. These results confirm a genetic polyandrous mating system in this species and suggest that fertilization pattern with respect to the sperm stores used is not random.

Keywords: sperm storage; sperm genetic diversity; mating system; sperm competition

1. INTRODUCTION

Polyandry is widespread in many taxa (Birkhead & Møller 1998) and, consequently, the ejaculates of different males often compete to fertilize a female's ova (Parker 1970). Sperm competition is particularly likely in species where sperm from multiple males are stored by the female prior to fertilization. In a presumed adaptation to this selective pressure, males of some species are known to manipulate or displace sperm from rival males during copulation. For example, male damselflies use their modified penis to mechanically remove sperm from the female sperm storage organ before transferring their own sperm (Siva-Jothy & Hooper 1995), many birds remove sperm plugs left by previous males by cloacal pecking (Birkhead & Møller 1992), and tree crickets flush sperm from previous matings using their own ejaculates (Ono *et al.* 1989). Stored sperm may also be manipulated by the females themselves (Eberhard 1996). The number of males that contribute to sperm stores (hereafter termed 'genetic diversity of sperm') has been shown to range from one (Chapuisat 1998), through mixtures skewed towards one male (Hammond *et al.* 2001), to mixtures from many males (Siva-Jothy & Hooper 1995; Urbani *et al.* 1998). Where females possess multiple sperm-storage sites, it is often unclear which site provides the fertilizing sperm. In such systems, females have the potential to 'choose' where to store sperm and which sperm to use during fertilization (Eberhard 1996). The corresponding null model that females use sperm from all stores randomly could still generate fitness benefits over (random) single-male mating (e.g. Tregenza & Wedell 2002); however, this model has rarely been tested.

Female decapod cephalopods (squid and cuttlefish) possess either a single or double sperm receptacle—a sperm storage organ—in their buccal area, and often also have access to sperm in the form of spermatangia (the sperm mass ejected from spermatophores) deposited on their buccal surface or mantle by males (Hanlon & Messenger 1996). It has been suggested that females may draw on any, or all, of these sperm stores to fertilize their eggs (Drew 1911; Hanlon & Messenger 1996; Hanlon *et al.* 1999). Indirect evidence for the use of stored sperm to fertilize eggs comes from studies showing that multiple paternity is common in squid egg fingers (Shaw & Boyle 1997; Buresch *et al.* 2001; Shaw & Sauer 2004). However, to date, there is no direct genetic support for this hypothesis.

The annual spawning aggregation of thousands of the giant Australian cuttlefish *Sepia apama* presents the opportunity to observe their natural reproductive behaviour (Hall & Hanlon 2002). Female *S. apama* alternate mating with oviposition, and may mate with multiple males between oviposition events (Hall & Hanlon 2002; Naud *et al.* 2004). Mating occurs in the head-to-head position during which males flush water vigorously across the female buccal area prior to transferring spermatophores to this area (Hall & Hanlon 2002). Eggs are deposited singly after being extruded (and presumably fertilized) in a temporary chamber formed by the female's arms. Sperm are stored in two locations: (i) the spermatangia (in the buccal area) and (ii) the sperm receptacle (beneath the beak). It is not known whether these two sperm stores are used differentially, and (if so) why.

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The aim of this study was to use microsatellite DNA typing, histology techniques and *in situ* collection of eggs to investigate (i) the structure of the sperm stores; (ii) how many males contributed sperm to the stores of females; and (iii) the possibility of biased sperm use with regard to storage site.

2. MATERIAL AND METHODS

(a) *Field methods*

Sampling was conducted by SCUBA diving near Black Point (137°43.1′ E, 32°59.5′ S), Spencer Gulf, Australia (Hall & Hanlon 2002). To assess the effects of male flushing on the numbers of spermatangia remaining in the female buccal area, 21 mating pairs were gently separated immediately after the male had completed flushing and before spermatophore transfer. Females were taken to the surface (3–5 m depth), where the number of spermatangia in their buccal area (figure 1) was estimated rapidly before they were released back into the water. To assess the numbers of spermatangia in females that had completed mating, 33 females were chosen haphazardly from the mating aggregation and spermatangia number was estimated in the same manner.

To assess the genetic composition of stored sperm and the paternity of sperm successfully achieving fertilization, focal females were observed continuously on site for up to 90 min ($n=11$ females). Throughout this period we collected all eggs laid by the focal females ($n=22$ eggs for 10 females, methods as in Naud *et al.* 2004). After the observation period, females were caught and killed humanely in iced seawater. The spermatangia and sperm receptacle were removed for genetic analysis. All samples were fixed in 99% ethanol.

(b) *Histology methods and volume estimations*

Histological sections of the sperm receptacle were taken from 11 sexually active females collected at the study site in May 2000. Tissue was fixed in 10% buffered formalin and standard techniques were used to clean and embed the samples. The 5 μm sections were stained with haematoxylin and eosin.

Estimation of the volume of sperm contained in the sperm receptacle was obtained by calculating the volume of a sphere, with dimensions derived from longitudinal and transverse section of a receptacle and by multiplying this volume by two (there are two receptacles; see results in §3). To estimate the total volume of sperm contained in the spermatangia, we multiplied the volume of a cylinder (with dimensions of spermatangium) by the mean number of spermatangia found in the buccal area of the focal females.

(c) *Genetic analysis*

Total DNA was extracted from spermatangia using a standard SDS extraction buffer with 1% dithiothreitol (DTT) and proteinase K (Carter *et al.* 2000). The sperm receptacles were dissected separately, digested using a standard SDS extraction buffer with proteinase K, and then spun at 13 000 rpm for 10 min to separate the undigested 'male fraction' (sperm cells have been reported to be resistant to digestion by SDS; Carter *et al.* 2000) from the digested female tissue. The resulting pellet was then digested in SDS/DTT buffer, and both fractions, as well as adult and offspring tissue, were extracted following a modified CTAB (2% *N*-cetyl *N*,*N*,*N*-trimethylammonium bromide) protocol (Winnepenninckx *et al.* 1993). All samples were genetically typed at six microsatellite DNA loci by PCR amplification with species-specific primers (Shaw 2003; genetic typing methods as in Naud *et al.* 2004).

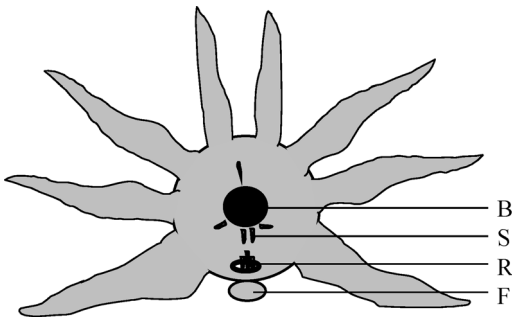

Figure 1. Location of sperm sources in the buccal area of a hypothetical female *Sepia apama*. The view is of the front of a female with all eight arms spread. A few spermatangia are illustrated as an example, both around the beak (B) and on the receptacles (R). S, spermatangia; and F, funnel.

The total number of different alleles at each locus that amplified from sperm source samples was divided by two (diploid). The minimum number of individuals having contributed sperm to this site was determined from the locus (or loci) with the highest minimum number of potential individuals. Alleles consistent with the female genotype were excluded before calculating the number of possible male genotypes in the sperm stores of the female concerned. Numbers of male genotypes are therefore conservative minimum estimates.

(d) *Statistical analysis*

Data were non-normally distributed, but met the assumptions for non-parametric tests, which were therefore used throughout. All probabilities were two-tailed. One female was discarded from the analysis of the origin of fertilizing sperm since there was mismatch with her presumed offspring's alleles. Observed and expected levels of heterozygosity at the microsatellite loci as well as exclusion probabilities were assessed using the program Cervus v. 2.0 (Marshall *et al.* 1998). Paternity by a specific source (matching of offspring to particular sperm stores) was excluded if the sperm source genotype did not share a non-maternal allele with the offspring at any one locus. F_{IS} values (Weir & Cockerham 1984) were calculated using the program Fstat v. 2.9.3.2 (February 2002).

3. RESULTS

(a) *Field data*

There was no difference between the numbers of spermatangia found around the buccal area of females sampled haphazardly on the spawning ground (mean ± s.d. = 16 ± 11, range 0–43, $n=33$) and those sampled after flushing (before spermatophore transfer; 20 ± 24.5, range 0–100, $n=21$; Mann–Whitney, $z=-0.737$, $p=0.461$).

(b) *Structure of the sperm stores*

The sperm were stored in a fleshy protuberance of oval shape located below the beak of the female (figure 1). Histological sections ($n=11$ protuberances from 11 females) revealed that each side of this protuberance is a receptacle formed by bulbs containing stored sperm (figure 2*a*). There were multiple spermatangia attached on top of the protuberance that contains the internal receptacles (figure 2*a*). On each side, the bulbs joined into a canal that opened to the buccal area and thus the two

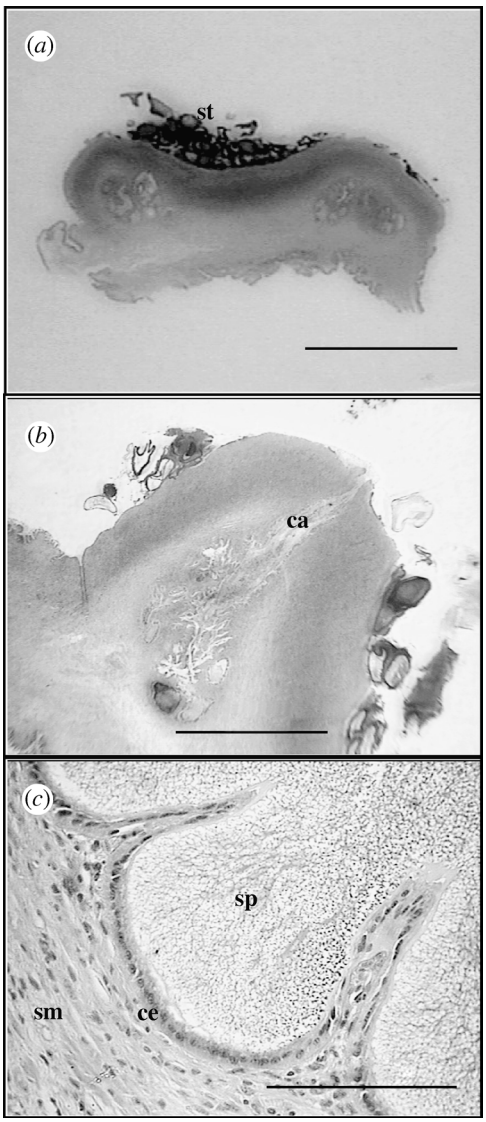

Figure 2. Histological sections of the sperm receptacles in *Sepia apama*. (*a*) Longitudinal section showing the two sperm receptacles. Note the spermatangia (st) on top of the receptacles. (*b*) Transverse section of one receptacle showing the canal (ca) opening to the buccal area. (*c*) Details of the bulbs of the sperm receptacle showing the simple columnar epithelium cells (ce), the sperm cells (sp) and the smooth muscle cells (sm); magnification ×250. Scale bars: (*a*) 8mm; (*b*) 4mm; (*c*) 100μm.

receptacles were physically separated (figure 2*b*). The inside of the bulbs was lined with a simple columnar epithelium and each bulb was surrounded by smooth muscle cells (figure 2*c*).

Estimated volumes of sperm found in the spermatangia and the receptacles were statistically comparable (mean volume ± s.d.; spermatangia, 36.2 ± 17.1 mm^3, $n=42$ from $n=42$ males; receptacles, 52.1 ± 60.8 mm^3, $n=9$ from $n=9$ females; Mann–Whitney, $z=-0.334$, $p=0.739$).

(c) *Population allele distribution*

The mean number of alleles per locus present in the study population was 10.7 (range 7–15, $n=203$). Comparisons between observed and expected heterozygosities and F_{IS} values indicated no significant deficit of heterozygotes either at individual locus or over all loci combined (randomization test, $F_{IS}=0.023$, $p=0.356$). Overall exclusion probabilities were 0.987 and 0.999 for exclusion without or with a known parent, respectively.

Table 1. Summary of the number of spermatangia (sptan) and genetic analysis of sperm storage for each female *Sepia apama* sampled.

female id	total sptan	number of genotypes recepta-cles[a]	number of genotypes sptan[a]	alleles different between receptacles (%)[b]
A	7	2+	2+	33
B	19	2+	4+	18
C	34	3+	3+	41
D	11	1+	2+	35
E	18	2+	3+	53
F	4	2+	2+	39
G	21	1+	5+	12
H	8	2+	3+	36
I	35	2+	2+	25
J	23	2+	3+	47
K	14	2+	2+	25
mean	17.6	1.9+ males	2.8+ males	33

[a] Minimum number of male genotypes determined from allelic diversity at a mean of four different microsatellite loci.
[b] Percentage of different alleles present in only one or the other of the two receptacles.

(d) *Genetic typing of the sperm receptacles and spermatangia*

A mean of 17.8 spermatangia (range = 4–35, $n=11$ females; table 1) were found in females that were observed *in situ* and collected for genetic analysis. Genetic diversity of sperm in the spermatangia was significantly higher than that in the sperm receptacles (mean minimum number of males ± s.d.: receptacles = 1.9 ± 0.5, spermatangia = 2.8 ± 1.0, Wilcoxon test, $z=-2.264$, $p=0.024$; table 1). The mean minimum number of male genotypes found in the combined spermatangia and receptacles of females was 3.4 males (range 2–5). The two sperm receptacles exhibited differences in their allelic profiles in every female tested, with a mean of 33% (range 11 to 53%) of alleles present in only one or the other of the two receptacles.

Out of the 22 offspring collected, paternal alleles were found among the female's sperm stores on 18 occasions (table 2). Paternal alleles were found more often in the spermatangia than the sperm receptacles (14–3, respectively, and both locations in one case, which was not used in the test; binomial test, $n=17$, $p=0.013$). However, the true level of statistical replication is the female, and when using one randomly chosen offspring per female, the difference was not significant (paternal allele found in: receptacles = 2, spermatangia = 8. Binomial test, $n=10$, $p=0.109$, power = 0.6597; table 2).

4. DISCUSSION

Our data represent, to our knowledge, the first account of the genetic diversity and use of sperm from multiple stores in any mollusc species. Indeed, with two notable exceptions in damselfly (Siva-Jothy & Hooper 1995, 1996), this seems to be the first such report for any taxon.

Previous studies have shown high rates of multiple mating in *S. apama* (Hall & Hanlon 2002), and

Table 2. Sperm sources origin of sperm used in fertilization in *Sepia apama*. sptan, spermatangia.

female	offspring	origin of fertilizing sperm[a]
A	1	recep
	2	recep/sptan
	3	recep
B	1	sptan
	2	?
C	1	sptan
	2	sptan
	3	sptan
D	1	sptop/sptan
	2	sptop/sptan
E	1	sptop
	2	sptop
F	1	recep
G	1	sptop
H	1	?
	2	?
	3	sptan
	4	sptop
I	1	sptan
	2	?
K	1	sptan
	2	sptop/sptan

[a] Origin of sperm fertilizing each egg (as determined genetically): recep, receptacles; sptan, spermatangia located in the buccal area; sptop, spermatangia located on top of the sperm receptacles; ?, unknown (origin of paternal sperm remained unassigned when all sperm sources were excluded genetically).

corresponding high rates of multiple paternity (67%) among eggs laid over time-scales of a few hours (Naud *et al.* 2004). Consequently, we expected to find several male genotypes expressed in the stored sperm of females. Our results confirm this; sperm diversity in all sperm stores combined was derived from 2 to 5+ males. This supports an earlier prediction of high probability of sperm competition prior to fertilization in this species (Naud *et al.* 2004) and is comparable with the number of males (2–4) contributing to fertilization of Loliginid squid egg strings (Shaw & Boyle 1997; Buresch *et al.* 2001; Shaw & Sauer 2004).

Few studies have characterized genetic diversity of stored sperm directly. Studies in ants have typically found sperm from only one male (as predicted by rare remating by most queens; Chapuisat 1998; Hammond *et al.* 2001; but see Fernández-Escudero *et al.* 2002). However, in damselflies, Siva-Jothy & Hooper (1995) found sperm from approximately three males in each female. Urbani *et al.* (1998) genotyped the sperm present in the spermathecae of female snow crabs (*Chionoecetes opilio*) mated in the laboratory and found the sperm of all possible mates (2–4) in seven out of eight cases. Our results support the emerging trend that where females mate with multiple males, they store sperm from these multiple mates.

We found that the genetic diversity of sperm in the spermatangia was significantly higher than that found in the receptacle. This finding is interesting in the context of flushing behaviour by males. This behaviour is thought to increase the likelihood of removal of fresh spermatangia from the buccal area, but unlikely to remove sperm from the sperm receptacles (Hanlon *et al.* 1999). Our data do not support this hypothesis. Numbers of spermatangia remaining in the female buccal area after flushing and before mating were not significantly different from those in haphazardly chosen females. A recent study also found no relationship between flushing time and fertilization success (Naud *et al.* 2004), but Hall & Hanlon (2002) noted that large males (which were usually the consorts) spent more time 'flushing' than small males. This raises questions about the function of this flushing behaviour. By contrast, in the damselfly, the bursa copulatrix (where males deposit sperm) contains sperm from fewer males than the spermathecae (ultimate site of sperm storage). This finding is consistent with the behaviour of male damselflies, which remove rival male sperm from the bursa during mating (Siva-Jothy & Hooper 1995). One hypothesis for the observed pattern in *S. apama* is that the function of flushing by males is not to remove spermatangia but rather to remove sperm from the receptacles. However, it is not possible to confirm or refute this hypothesis here.

The structure of the sperm receptacles has been studied in very few cephalopod species. In the common European cuttlefish, *Sepia officinalis*, two separate sperm receptacles open via pores on the ventral surface of the female's buccal area (Hanlon *et al.* 1999). The internal structure of the receptacles appears similar to that of *S. apama*, with bulbs surrounded by muscle cells (Hanlon *et al.* 1999). Drew (1911) described the gross anatomy of the single sperm receptacle of the squid *Loligo pealeii* as being similar to a compound alveolar gland, and also noted the presence of muscle cells around it. Interestingly, during a study on yellow dung fly, Hellriegel & Bernasconi (2000) showed that sperm distribution in two storage sites of females, which were anaesthetized after copulation, differed from that in non-anaesthetized females. They suggested that female muscular activity may affect storage and differential placement of competing ejaculates after copulation (Hellriegel & Bernasconi 2000). Whether each of the twin sperm receptacles in *S. apama* has separate innervation, and could therefore be controlled in the same manner, is not known, but finding different compositions of male alleles (indicating different mixtures of sperm) in the two chambers of individual females may be anecdotal support for such independent functioning.

Our study confirms that sperm from both the spermatangia and the receptacles are used by female *S. apama* for fertilization. Although the volumes of sperm available in the receptacles and the spermatangia of *S. apama* were comparable, sperm from the spermatangia fertilized the egg four times more often than sperm from the receptacles. Such a pattern might result if sperm from spermatangia in the buccal area fertilize their eggs (these sperm would have first access to the egg as the female places it there), but that when spermatangia sperm sources are low, sperm from the receptacles are used. Alternatively, the structure of the sperm stores may be responsible for the differential fertilization success: sperm can be obtained from any point on the surfaces of the spermatangia, while the sperm contained in the receptacles must exit through small openings before being available for fertilization.

It is probable that the receptacles extend sperm viability and thereby provide an important long-term sperm store for females. The spawning aggregation lasts for up to five months and individual cuttlefish are thought to spawn multiple times over a period of several weeks (up to a few

months; Hall 2002). *S. apama* is found throughout southern Australia, yet only one dense spawning aggregation is known. In this context, the abundance of spermatangia in the buccal area of females may be characteristic of the dense spawning aggregation studied, but not of *S. apama* spawning at low population densities. Females spawning on sites with low conspecific density may encounter and mate with few males and hence rely on sperm stored in the receptacles to fertilize most of their eggs. Although sperm within spermatophores are long lived (many weeks), the longevity of sperm that have been released from spermatangia attached to the buccal surface of females is of the order of days (M.-J. Naud and J. N. Havenhand, unpublished work). It seems probable that sperm contained in the receptacles would have far greater longevity, as in the squid *L. pealeii* (Drew 1911; Maxwell & Hanlon 2000).

In summary, the results of this study confirm that female *S. apama* have access to, and use, sperm from several males stored in two sites to fertilize their eggs. The observation that most eggs were fertilized by sperm originating from the spermatangia rather than from the receptacles, but that the respective sperm volumes were comparable, indicates a bias in fertilization success with regard to sperm store origin. Determining whether this fertilization bias results from active female manipulation of stored sperm (i.e. post-copulatory choice) or from simple physical constraints on sperm access to eggs will be an interesting future line of study. Only one equivalent study exists in the literature (Siva-Jothy & Hooper 1996), and therefore it is difficult to assess how general our findings are. Nonetheless, we have shown clearly the biased use of sperm from different sperm sources in this population of *S. apama*, suggesting the potential for post-copulatory female mate choice in this species.

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